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KINETICS OF THE EFFECTS OF OXYGEN ON LYOPHILIZED *SERRATIA MARCESCENS*

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ABSTRACT

Dried Serratia marcescens (ATCC strain 14041) were exposed to various partial pressures of oxygen and nitrogen. The colony-forming ability of the organisms was rapidly destroyed during exposure to oxygen but unimpaired by exposure to purified nitrogen. The degree of inactivation depended upon temperature, time, and the partial pressure of oxygen regardless of whether pure oxygen or dry air was used. The inactivation by oxygen followed the expression $-\ln N/N_o = k[O_2]^{1/3}t^{1/2}$ where N_o and N are the number of viable organisms before and after exposure respectively, $[O_2]$ is oxygen concentration, t is time, and k the rate constant. At 25 C, k was 276±36 $moles^{-1/3}cc^{1/3}hr^{-1/2}$ for oxygen pressures between 5.5 and 258 torr. In the temperature range between -78 and 40 C, the rate constant may be expressed as $k = 10^{5.95\pm0.42}\exp[(-430\pm26)cal/RT]moles^{-1/3}cc^{1/3}hr^{-1/2}$.

I. INTRODUCTION

Rogers[1] was one of the first investigators to recognize the lethal effects of oxygen on lyophilized organisms. Later Naylor and Smith[2] reported results that substantiated Rogers' results. These investigators reported that survival was highest for organisms stored under vacuum and lowest for those stored in air or oxygen. Atmospheres of nitrogen, hydrogen, and carbon dioxide yielded intermediate results. Scott[3] indicated that the effect of the atmosphere upon the survival of dried bacteria depended upon the nature of the suspending medium and its moisture content. Recently Lion and Bergmann[4,5] listed numerous substances that protect lyophilized Escherichia coli against the lethal effects of oxygen. Lion[6] suggested that a prerequisite for effective protection against oxygen in the dry state is the accumulation of the solute around the bacteria, which he assumed to have occurred during lyophilization. Benedict et al.[7] reported that atmospheric oxygen killed 95% of dried Serratia marcescens in 10 minutes, that certain reducing agents prevented the action of the oxygen, and that humidity seemed to play no role in the phenomenon.

The nature of the adverse effect of oxygen on dried bacteria is still not understood. Lion, Kirby-Smith, and Randolph[8] showed that free radical production, measured by an increase in the relative electron paramagnetic resonance (EPR) signal, occurred when dry E. coli was stored in the presence of oxygen. S. marcescens was shown to exhibit the same phenomenon by Dimmick, Heckly, and Hollis.[9] The EPR studies were extended by Heckly et al.[10] to include the effects of moisture, selected protective additives, and other environmental factors. Lion and Avi-Dor[11] showed that nicotinamide adenine dinucleotide (NADH) oxidase activity was inhibited in lyophilized E. coli after exposure to oxygen. Hess[12] has recently reported that S. marcescens were rapidly inactivated when aerosolized in air but that their colony-forming ability was almost unimpaired when the organisms were aerosolized in relatively pure nitrogen.

This investigation was undertaken to determine (i) a rate expression for the inactivation of dried S. marcescens by oxygen, (ii) the Arrhenius parameters for inactivation, and (iii) whether the lethal effects of oxygen observed when water suspensions of S. marcescens are aerosolized[12] are similar to those observed when lyophilized organisms are exposed to oxygen.

II. MATERIALS AND METHODS

The methods for growing the S. marcescens (Fort Detrick strain 8UK, ATCC strain 14041), determination of viable cell populations, lyophilization, and rehydration were described previously.[18] Triply washed bacterial suspensions containing about 2×10^{10} organisms per ml were routinely used. From 45 to 70% of the viable cell populations in the parent suspensions survived lyophilization and these dried materials were used for the various studies.

The oxygen (Southern Oxygen Company, Hagerstown, Md.) used was predried by passing it through a trap of activated silica gel at dry ice temperatures. The dried oxygen was stored in 2-liter flasks on the high vacuum manifold and used as needed. Room air was dried and stored in the same manner prior to use. Nitrogen containing less than 10 ppm of water and less than 2 ppm of oxygen was obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey, in sealed pyrex bulbs. The nitrogen was further treated to reduce the oxygen content by allowing it to remain in contact with a mirror of vacuum-distilled sodium metal for a few days. Gas pressures were measured with a McLeod gauge or mercury manometer. Contact of mercury vapor with the test organisms was avoided by using cold traps.

The bacterial suspensions were lyophilized at less than 10^{-5} torr in 20-ml ampoules containing glass beads of 4 mm diameter. Immediately preceding exposure, the ampoules were slightly jarred to break up the dried caked material with the glass beads. At the beginning of this study, dried samples were vigorously shaken to determine whether this would increase the exposure surface of the dried material, thus resulting in a possible increase in the rate of inactivation during exposure. Vigorous shaking in the presence of the glass beads led to inactivation of a substantial percentage of the organisms, but with gentle jarring the mechanical destruction was negligible. In both cases the remaining viable organisms decayed at the same rate when exposed to a given partial pressure of oxygen. Organisms dried in the absence of the glass beads, however, showed slower and nonreproducible inactivation rates during exposure. During the exposure studies at 25 C, ampoules containing the dried organisms were immersed in a water bath thermostatically controlled at 25 C (±0.5).

Dried organisms to be exposed to dry air at temperatures below 25 C were cooled to the desired temperature before the air was introduced. After the 1-hour exposure, the ampoules were evacuated and warmed to 25 C before rehydration.

III. RESULTS

Some survival vs. time data obtained by exposing lyophilized S. marcescens at various pressures of oxygen, dry air, and nitrogen are plotted in Figure 1. No loss in viability could be detected when the dried organisms were held under vacuum (pressures less than 10^{-5} torr) for periods up to 3 hours. The semi-logarithm plot of N/N_o vs. time indicates that the decay rate decreases as time increases for a given partial pressure of oxygen. Since no pressure changes were detected during these studies, it was concluded that oxygen inactivation results reported in this paper are representative of the effects of a large excess of oxygen compared with those of the amount required for the inactivation observed during the exposure period.

Figure 2 shows the dependence of viability upon the partial pressure of oxygen after ½- and 1-hour exposures at 25 C. The extreme sensitivity of the dried suspensions to low oxygen pressures was pronounced; about 75% of the organisms were inactivated in ½ hour by oxygen at a pressure of 10 torr. Also, losses in viability after exposure to dry air were identical to those obtained with pure oxygen when both were normalized to the same partial pressures of oxygen.

Figure 3 gives another representation of the inactivation curves given in Figure 1. Plots of log N/N_o vs. $(\text{time})^{\frac{1}{2}}$ fitted all the survival data well. It should be noted that the straight lines obtained for the various partial pressures of oxygen extrapolate to $N/N_o = 1$ at $t = 0$. A rate expression for the oxygen inactivation of lyophilized S. marcescens can be written: $-\ln N/N_o = K t^{\frac{1}{2}}$ where K is a pseudo rate constant dependent upon the partial pressure of oxygen, and t is time. The pseudo rate constants, K's, for all the oxygen inactivation data were obtained by determining the slopes of log N/N_o vs. $(\text{time})^{\frac{1}{2}}$ plots by the least-squares method. These data are given in Table 1.

The pseudo rate constant is related to the oxygen concentration by the expression:

$$K = k[O_2]^n$$

or

$$\log_{10} K = n \log_{10}[O_2] + \log_{10} k$$

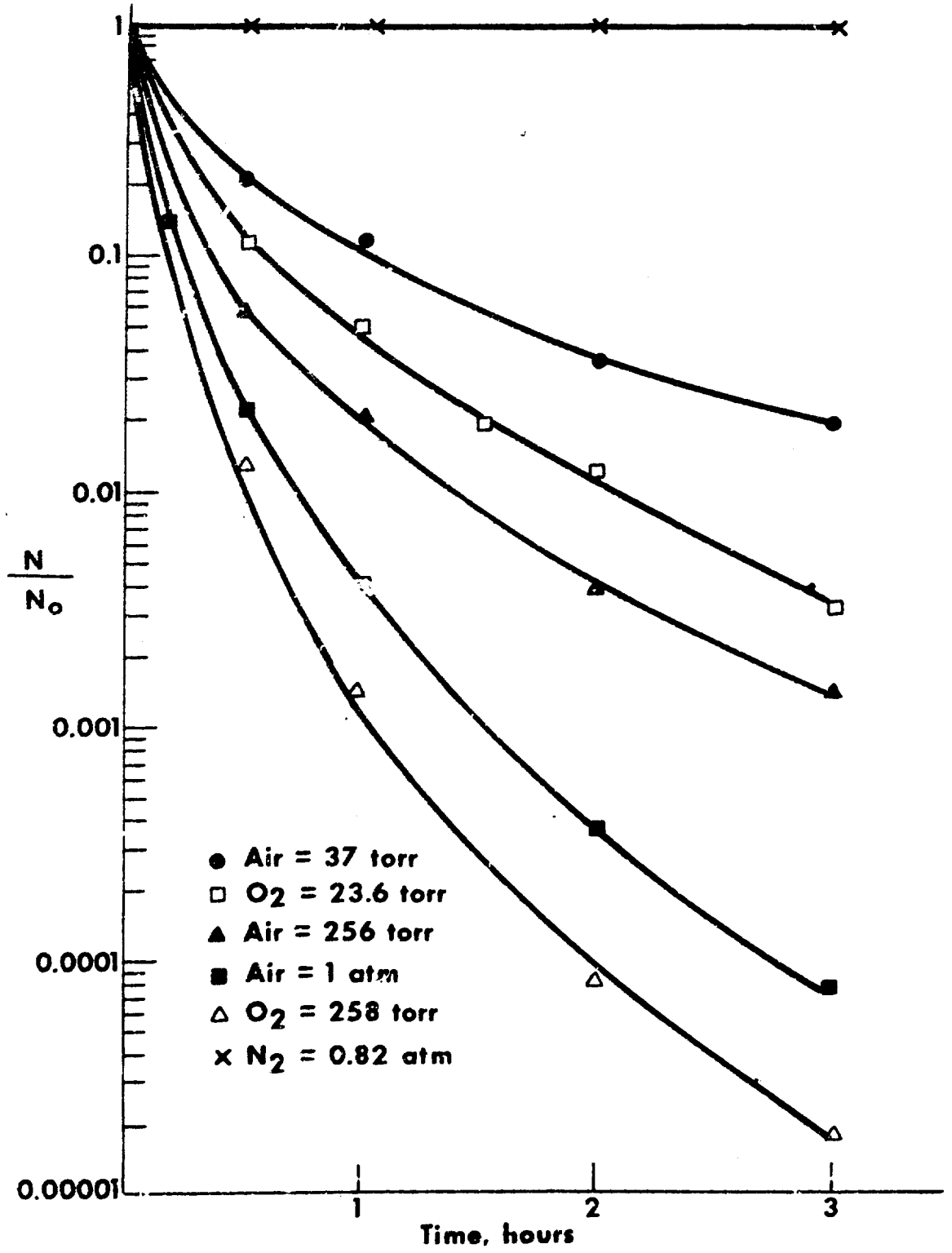

Figure 1. Semi-Log Plot of Survival vs. Time after Exposure of Lyophilized S. marcescens to Various Pressures of Oxygen, Dry Air, or Purified Nitrogen. N_o and N are the number of viable organisms before and after the exposure respectively.

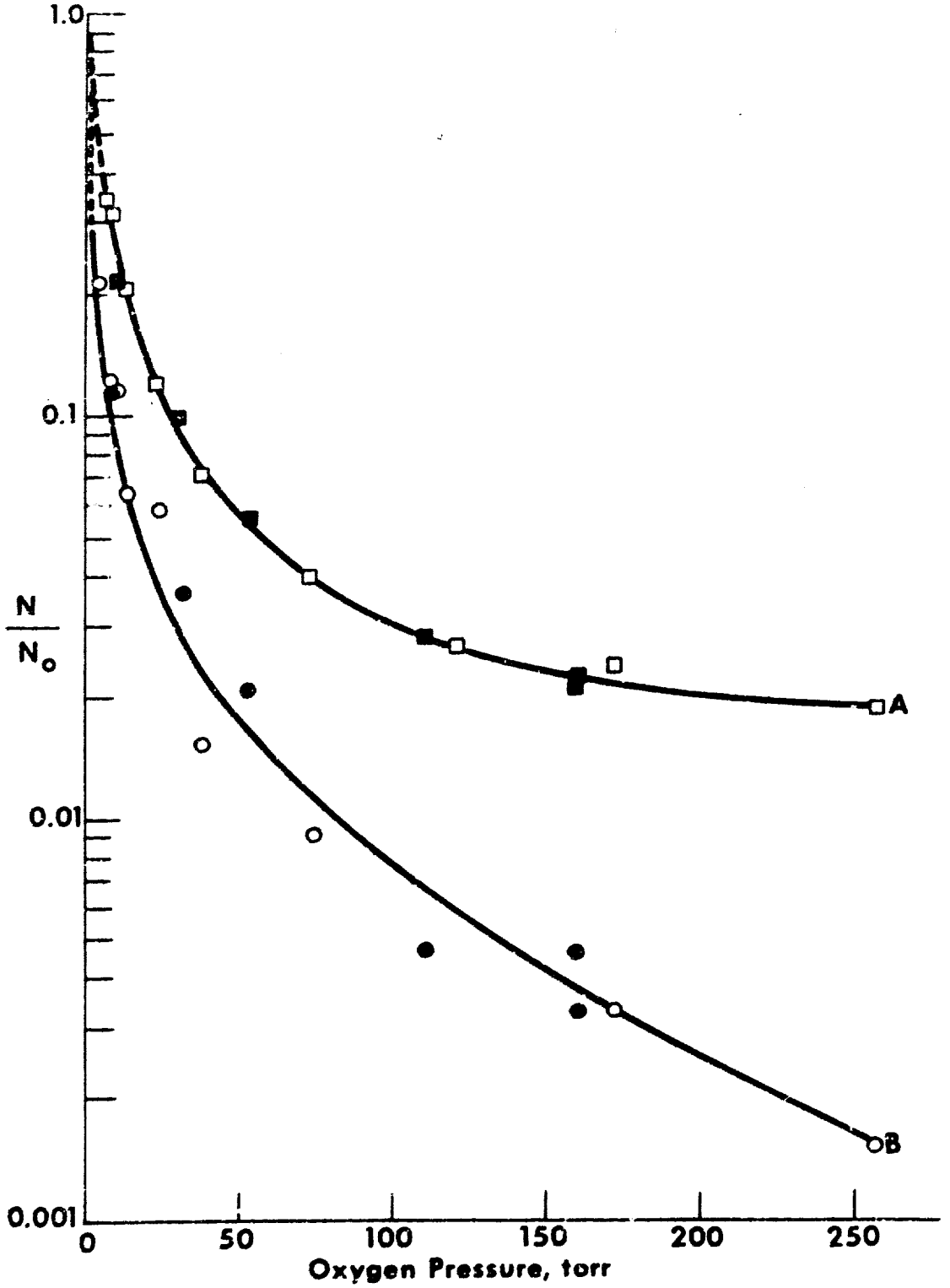

Figure 2. Semi-Log Plot of Survival vs. Oxygen Pressure after ½ (Line A) and 1 Hour (Line B) Exposures to Oxygen or Partial Pressures of Oxygen in Dry Air; N_o and N are the Number of Viable Organisms before and after Exposure Respectively. Open circles and squares, pure oxygen: closed circles and squares, partial pressure of oxygen in dry air.

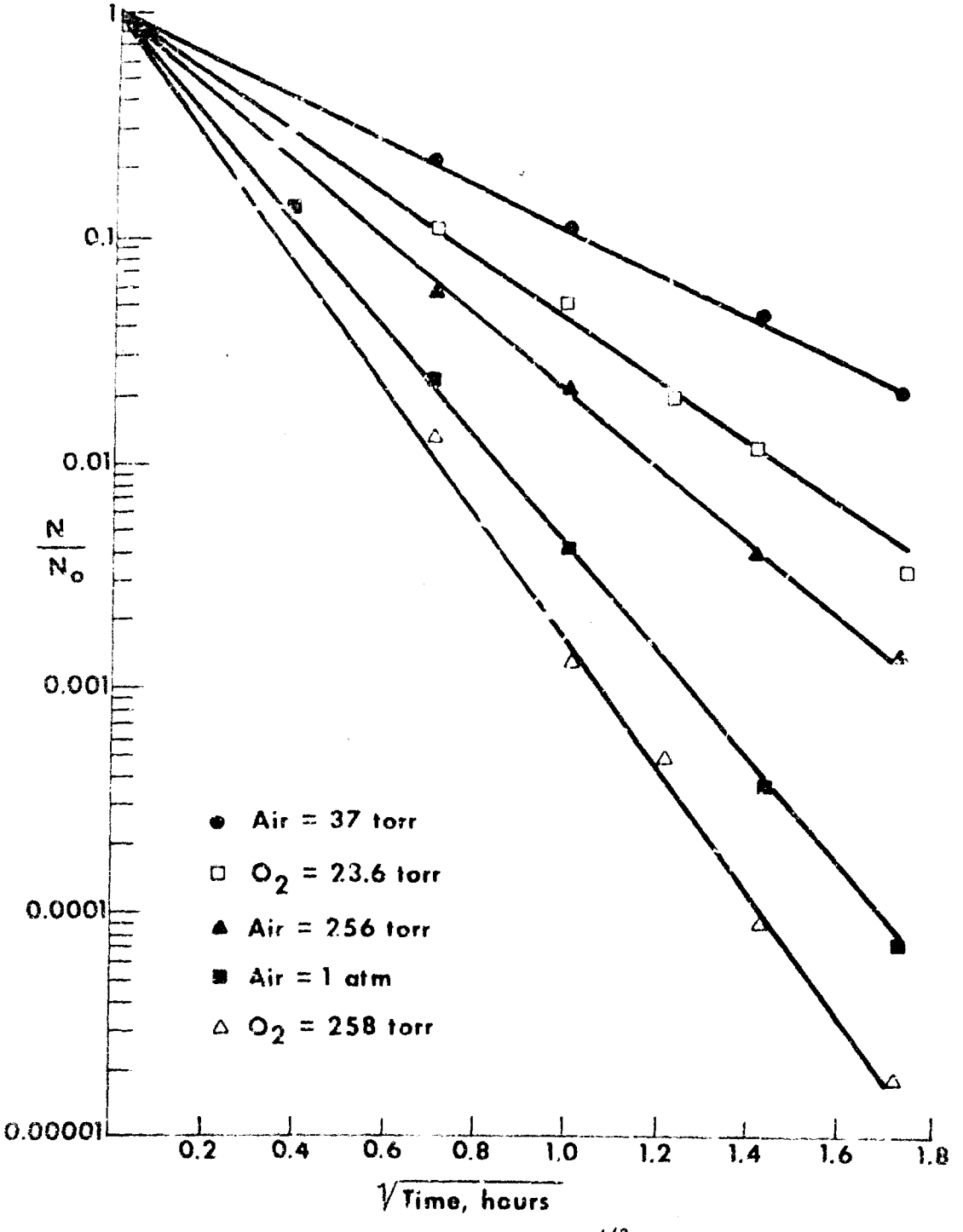

Figure 3. Semi-Log Plot of Survival vs. $(\text{Time})^{1/2}$ after Exposure of Lyophilized S. marcescens to Various Pressures of Oxygen or Dry Air. N_o and N are the number of viable organisms before and after the exposure respectively.

TABLE 1. KINETIC DATA FOR THE INACTIVATION OF SERRATIA MARCESCENS BY OXYGEN

Oxygen Pressure, torr	Oxygen Concentration,[a] 10^{-7} mole/cc	K Pseudo, $hr^{-1/2}$	k, $moles^{-1/3}cc^{1/3}hr^{-1/2}$
258	139	6.51	271
172	92.5	5.50	261
160[b]	86.1	5.17	252
159[b]	85.5	6.13	300
121	65.0	5.27	283
110[b]	59.2	4.79	275
71.6	38.5	4.15	264
53.7[b]	28.9	3.85	271
37.7	20.3	4.28	337
30.8[b]	16.6	3.02	258
23.6	12.7	3.31	306
11.0	5.92	2.11	251
8.0	4.30	1.85	245
7.8[b]	4.20	2.29	306
5.5	2.96	1.78	267

a. Calculated by using the ideal gas law.
b. Dry air used as source of oxygen.

where n is the order of the rate expression in oxygen and k is the rate constant. A plot of the $\log_{10}$ of the pseudo rate constant, K, vs. $\log_{10}$ of the oxygen concentration is given in Figure 4. A least-squares fit of the data yields a slope of 0.328±0.022, which is approximately 1/3. Therefore, the inactivation by the oxygen can be expressed as follows:

$$-\ln N/N_o = k[O_2]^{1/3}t^{1/2}$$

where N_o and N are the number of viable organisms before and after exposure respectively, $[O_2]$ is the oxygen concentration, t is time, and k is the rate constant, which was found to be 276±36 $moles^{-1/3}cc^{1/3}hr^{-1/2}$ at 25 C.

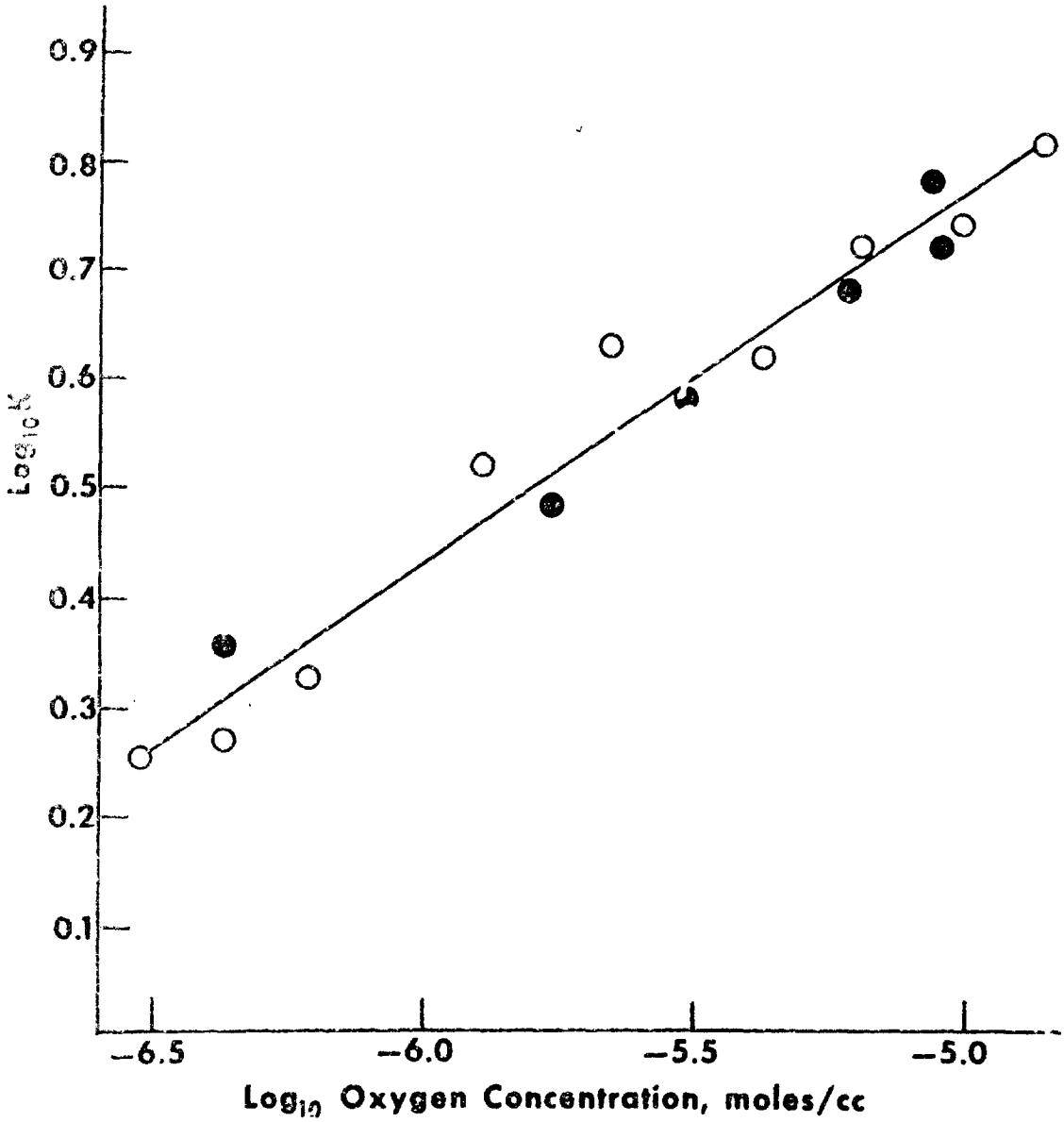

Figure 4. Log_{10} of the Pseudo Rate Constant, K, vs. Log_{10} of the Oxygen Concentration. Open circles, pure oxygen; closed circles, dry air.

Dried organisms were exposed to dry air at atmospheric pressure for 1 hour at -78, -15, 0, and 40 C. The values obtained for the viability losses were corrected for the losses that occur when dried organisms under vacuum are subjected to the various temperatures for 1 hour.[13] The corrected values of N/N_o were then used to estimate the rate constant, k, for the various temperatures. The results are plotted as the Arrhenius function in Figure 5. A line determined by the least-squares method through the points for -78 to 40 C yields:

$$k = 10^{5.95\pm0.42}\exp[(-430\pm26)\text{cal}/RT]\text{moles}^{-1/3}\text{cc}^{1/2}\text{hr}^{-1/2}$$

IV. DISCUSSION

Lyophilized S. marcescens rapidly become nonviable when exposed to oxygen or dry air. The degree of this inactivation was found to be dependent upon time, temperature, and the partial pressure of oxygen. Lion and Bergmann[4] reported results on the inactivation of lyophilized E. coli by oxygen. Their qualitative results are in agreement with those of this work, but they did not report a rate expression for the inactivation process. However, their survival vs. time data fit the log N/N_o vs. $(\text{time})^{1/2}$ relationship. Dunklin and Puck[14] reported decay data of airborne organisms and showed that the decay was more dependent on relative humidity (RH) in the presence than in the absence of added solutes. They considered the inactivation process as consisting of an initial rapid first-order rate process followed by a slower one. However, their data can be represented with equal success by a log N/N_o vs. $(\text{time})^{1/2}$ plot indicating that two different decay processes are not necessary to explain their data. Hess[12] has reported a study on the inactivation of aerosolized S. marcescens in atmospheres containing various oxygen contents. Figure 6 gives a comparison of the inactivation reported by Hess for aerosolized organisms with the result of the present work. There appears to be no marked difference in the degree of inactivation after ½ hour as a function of the partial pressure of oxygen in the two systems. It should be noted that Hess' data were generated at 40% RH but 0% RH was used in this work, hence direct comparison is not completely valid. However, preliminary studies conducted in this laboratory on the inactivation of lyophilized S. marcescens by oxygen in humidified air indicate that the degree of inactivation of washed lyophilized organisms was essentially independent of RH between 0% and 85%, but when organisms were lyophilized from suspensions containing 0.05% NaCl there was a marked RH dependence upon the degree of inactivation by air. The results of these experiments will be reported elsewhere later. Hess pointed out that the inactivation by oxygen was the major cause of death when S. marcescens were subjected to aerosolization in atmospheres of dehydrating levels of RH. Webb[15]

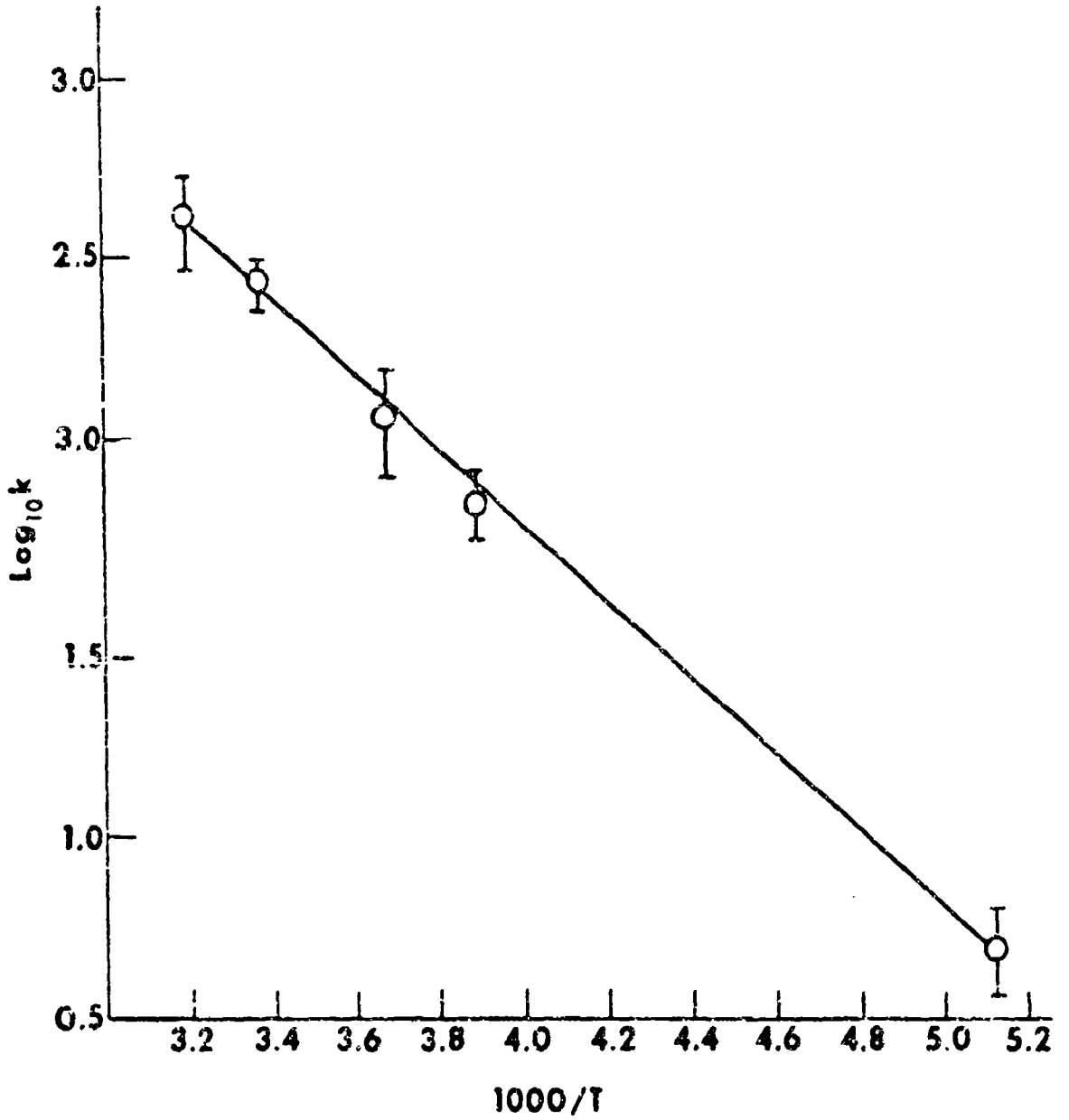

Figure 5. Arrhenius Plot for the Inactivation of S. marcescens by Oxygen. On the abscissa, T is in degrees Kelvin.

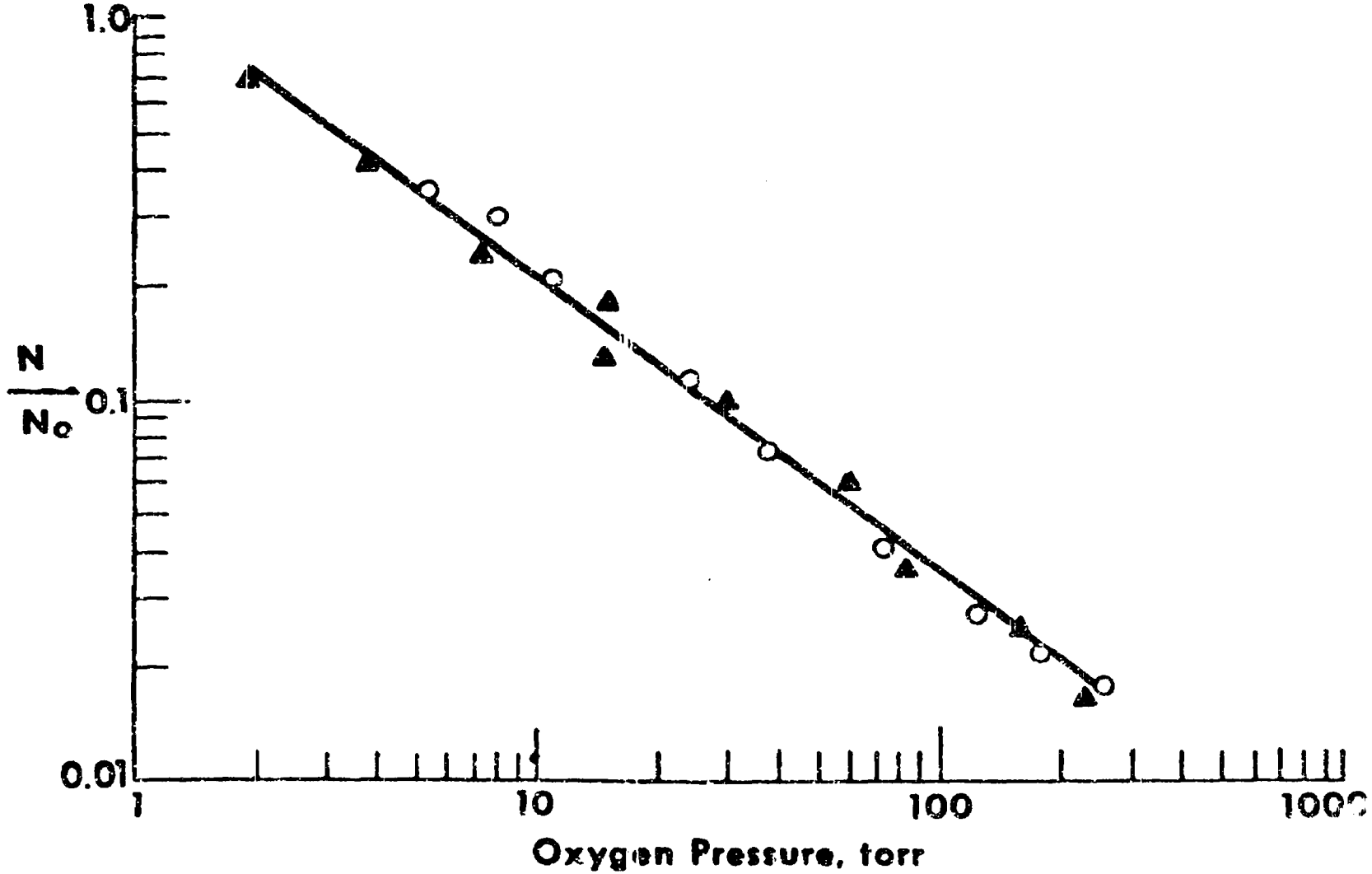

Figure 6. Log-Log Plot of Survival of S. marcescens vs. Partial Pressure of Oxygen. N_o and N are the number of viable organisms before and after the stress. Open circles, this work, lyophilized organisms after ½ hour exposure; closed triangles, aerosolized organisms after 32 minutes.[12]

suggested that the lethal effects in the aerosol are due to collapse of protein structures upon dehydration and later reported additives that were capable of replacing cellular water thus increasing the survival of aerosolized S. marcescens.[16] It appears that Webb overlooked the inactivation by oxygen; this is understandable when one considers the extreme sensitivity of dehydrated organisms to even small oxygen concentrations (Fig. 2).

Some remarks about kinetic interpretation of these data seem warranted at this time. The amount of oxygen required for inactivation is small because no pressure changes were detected during the exposures (pressure changes >5% could have been detected). Lion, Kirby-Smith, and Randolph[8] came to a similar conclusion in their work on the inactivation of E. coli by molecular oxygen. The role played by molecular oxygen in the inactivation process is unknown and it would be meaningless at this time to formulate a mechanism only on the basis of rate data. The preliminary kinetic studies reported in the present work as well as those of Lion and Bergmann show that the inactivation process is not simply pseudo first-order. In fact, the integrated rate expression found here suggests that a chain mechanism involving radicals[8,10] is probably occurring during the inactivation process.

It would be of interest to compare the integrated rate expression reported here with that of other atmospheric gases that impair viability. More kinetic data in this area should be useful in explaining the air sterilization of microorganisms.

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13 ABSTRACT

Dried Serratia marcescens (ATCC strain 14041) were exposed to various partial pressures of oxygen and nitrogen. The colony-forming ability of the organisms was rapidly destroyed during exposure to oxygen but unimpaired by exposure to purified nitrogen. The degree of inactivation depended upon temperature, time, and the partial pressure of oxygen regardless of whether pure oxygen or dry air was used. The inactivation by oxygen followed the expression $-\ln N/N_o = k[O_2]^{1/3}t^{1/2}$ where N_o and N are the number of viable organisms before and after exposure respectively, $[O_2]$ is oxygen concentration, t is time, and k the rate constant. At 25 C, k was 276 ± 36 moles$^{-1/3}$cc$^{1/3}$hr$^{-1/2}$ for oxygen pressures between 5.5 and 258 torr. In the temperature range between -78 and 40 C, the rate constant may be expressed as $k = 10^{5.95 \pm 0.42}\exp[(-430 \pm 26)\text{cal}/RT]$ moles$^{-1/3}$cc$^{1/3}$hr$^{-1/2}$.

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